Effects of Oral Administration of *N*-Acetyl-L-cysteine: A Multi-Biomarker Study in Smokers

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Abstract

N-Acetyl-L-cysteine (NAC) has been shown to exert cancer-protective mechanisms and effects in experimental models. We report here the results of a randomized, double-blind, placebo-controlled, Phase II chemoprevention trial with NAC in healthy smoking volunteers. The subjects were supplemented daily with 2 × 600 mg of oral tablets of NAC ($n = 20$) or placebo ($n = 21$) for a period of 6 months, and internal dose markers [plasma and bronchoalveolar lavage (BAL) fluid cotinine, urine mutagenicity], biologically effective dose markers [smoking-related DNA adducts and hemoglobin (Hb) adducts], and biological response markers (micronuclei frequency and antioxidants scavenging capacity) were assessed at both pre- and postsupplementation times (T_0 and T_1, respectively). Overall, the internal dose markers remained unchanged at T_1 as compared with T_0 in both NAC and placebo groups. When quantifying the biologically effective dose markers, we observed an inhibitory effect of NAC toward the formation of lipophilic-DNA adducts (5.18 ± 0.73 *versus* 4.08 ± 1.03/10^8 nucleotides; mean ± SE; $P = 0.05$) as well as of 7,8-dihydro-8-oxo-2′-deoxyguanosine adducts in BAL cells (3.9 ± 0.6 *versus* 2.3 ± 0.2/10^5 nucleotides; $P = 0.003$). There was no effect of NAC on the formation of lipophilic-DNA adducts in peripheral blood lymphocytes or polycyclic aromatic hydrocarbon-DNA adducts in mouth floor/buccal mucosa cells or 4-aminobiphenyl-Hb adducts. Likewise, quantification of the biological response markers showed an inhibitory effect of NAC on the frequency of micronuclei in mouth floor and in soft palate cells (1.3 ± 0.2 *versus* 0.9 ± 0.2; $P = 0.001$) and a stimulating effect of NAC on plasma antioxidant scavenging capacity (393 ± 14 *versus* 473 ± 19 μM Trolox; $P = 0.1$) but not on BAL fluid antioxidant scavenging capacity. We conclude that NAC has the potential to impact upon tobacco smoke carcinogenicity in humans because it can modulate certain cancer-associated biomarkers in specific organs.

Introduction

The aminothiol NAC[2] has been in clinical practice since the years 1960s (1–4). Initially, NAC was introduced as a mucolytic agent for the treatment of respiratory diseases, *e.g.*, chronic bronchitis and cystic fibrosis (2, 5–7). In the late 1970s, NAC was recognized as an antidote for the therapy of acute acetaminophen intoxication (8, 9). More recently, NAC has been brought into use for preventing or attenuating pulmonary oxygen toxicity, adult respiratory distress syndrome, and influenza-like symptomatology and treating pulmonary obstructive diseases (10–17). The diverse pharmacological applications of NAC are inherent in the multifaceted chemical properties of its constituent, cysteinyl thiol, which enable NAC to act as a nucleophile as well as a scavenger of reactive oxygen species (14, 18–21). Because of these nucleophilic and antioxidant properties, NAC has also been proposed as a potential chemopreventive agent (13, 15, 21–23). Thus far, NAC has been reported to inhibit a variety of histopathological, cytological, clastogenic, metabolic, and molecular alterations induced by various mutagens and/or carcinogens in experimental rodent models (3, 21, 24–35). These inhibitory effects of NAC have been ascribed to its ability to serve as an analogue of cysteine as well as a precursor of reduced glutathione (GSH), to enhance the activities of glutathione *S*-transferases, glutathione peroxidase, glutathione reductase, NADH- and NAD(P)H-quinone reductase, and probably, to promote DNA repair by protecting ADP-ribosyltransferase activity (24, 36–47).

In chemoprevention trials, quantification of intermediate biomarkers can shed light on the chain of events pre- and postadministration of an agent of interest (48). For instance, internal/biologically effective dose markers can reflect the concrete/integrated exposure to carcinogens, or biological response markers can represent the event, which give rise to carcinogenesis both before and after the agent is administered. In the present study, we investigated the chemopreventive effects of NAC in humans by applying a multi-biomarker approach quan-

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[2] The abbreviations used are: NAC, *N*-acetyl-L-cysteine; BAL, bronchoalveolar lavage; Hb, hemoglobin; 4-ABP, 4-aminobiphenyl; 8-OH-dG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; PBL, peripheral blood lymphocyte; PAH, polycyclic aromatic hydrocarbon; MFC, mouth floor cell; BMC, buccal mucosa cell; SPC, soft palate cell; TEAC, Trolox equivalent antioxidant capacity; GC-MS, gas chromatography-mass spectrometry.

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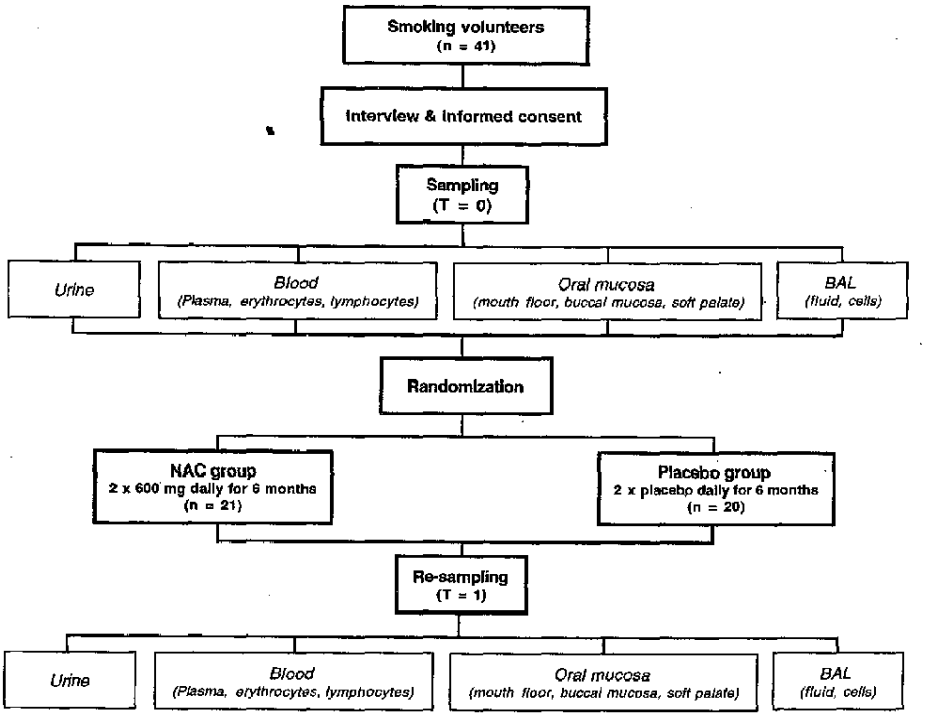

Fig. 1. Flow diagram of the study.

tifying the intermediate markers of exposure and effects induced by cigarette smoking. In a double-blind, placebo-controlled fashion, NAC was supplemented p.o. for a period of 6 months to healthy smokers, and internal dose markers (plasma and BAL fluid cotinine, and urine mutagenicity), biologically effective dose markers (smoking-related DNA adducts, oxidative DNA damage and Hb adducts), and biological response markers (frequency of micronuclei and antioxidants scavenging capacity) were assessed at both pre- and postintervention times. The laboratory assays used to quantitate the above-mentioned markers were as follows: (*a*) RIA for plasma and BAL fluid cotinine; (*b*) mutagenicity assay for urine mutagenicity; (*c*) gas chromatography-mass spectrophotometry assay for 4-ABP-Hb adducts; (*d*) nuclease P1 enriched ^{32}P-postlabeling assay for lipophilic-DNA adducts as well as for 8-OH-dG adducts in PBLs and BAL cells; (*e*) immunohistochemical peroxidase assay for PAH-DNA adducts in MFCs and BMCs; (*f*) micronucleus assay for cytogenetic analyses in MFCs and SPCs; and (*g*) TEAC assay for plasma and BAL fluid antioxidant scavenging capacity.

Materials and Methods

Study Population. Forty-one healthy volunteers who could not quit smoking were recruited by advertising in the national newspapers. Each participant was briefed about the study protocol, signed an informed consent, and filled out a comprehensive questionnaire seeking information on age, sex, occupation, and lifestyle including smoking and drinking behaviors, fresh fruit and vegetable consumption, vitamin supplementation, medical history of disease, and family history of cancer. The study population was randomly divided into two groups to be treated with NAC ($n = 21$) and placebo ($n = 20$), respectively (Fig. 1). Detailed characteristics of the study population are listed in Table 1. The study was approved by the Medical Ethical Commission of The Netherlands Cancer Institute.

Table 1 Characteristics of the study population[a]

	NAC group	Placebo group
Number	21	20
Sex (male/female)	6/15	8/12
Age	42 ± 2.3	44 ± 1.9
Cigarettes/day	24.8 ± 2.0	28.5 ± 3.1
Tar/day (mg)	335.0 ± 46.1	417.4 ± 56.9
Nicotine/day (mg)	29.3 ± 5.0	36.9 ± 6.1
Smoking years	25 ± 1.8	27 ± 1.8
Pack years	31.9 ± 3.7	39.8 ± 5.8

[a] Results are expressed as mean ± SE.

Study Protocol. Participants were instructed to strictly maintain their lifestyle and in particular their smoking behavior throughout the intervention period. The randomly assigned NAC or placebo groups were treated p.o. with two daily doses of NAC (600 mg each tablet; Zambon Group, Vicenza, Italy) or placebo for a period of 6 months in a double-blind fashion. The analysis was intention to treat, and there were no losses to follow up. Urine, peripheral blood, oral mucosa, and BAL of individuals were sampled at both pre- and postintervention times. To acquire the information on the side effects of NAC/placebo supplementation and to check the compliance with the study protocol, all participants filled out a weekly self-administered questionnaire during the course of the intervention and were also interviewed at the end of the intervention period.

Urine. Twenty-four-h urine was collected in polyethylene bottles. Density and pH were measured, and subsequently, 500 ml of the agitated sample were filtered through XAD-2 nonpolar

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resin according to the method of Yamasaki and Ames (49). The resulting concentrate was dissolved in DMSO (5 ml) and preserved at −20°C until further analysis.

Peripheral Blood. Thirty ml of venous blood was drawn into heparinized Venoject®II tubes (Terumo Europe N.V., Leuven, Belgium). Plasma, erythrocyte, and lymphocyte fractions were separated by applying standard centrifugation procedures (50, 51) and subsequently stored at −70°C until further analysis.

BAL. BAL was performed as described earlier (52). Briefly, under local anesthesia, a fiber bronchoscope was introduced into right middle lobe bronchus, and nine aliquots (20 ml each) of sterile 0.15 M NaCl were instilled consecutively with a dwelling time of 3 min. The recovered fluid was collected in plastic tubes placed on ice, and after total and differential cell counts, it was pelleted by centrifugation and kept at −70°C until further analysis.

Oral Mucosa. MFCs, BMCs, and SPCs were harvested by brushing precisely the floor of the mouth, the middle part of the inside of the cheek, and the center of the soft palate, respectively, with an interdental brush (Lactona, Almere, the Netherlands). The brushes were stirred in 10-ml Greiner tubes (Greiner Labortechnik, Frickenhausen, Germany) prefilled with PBS (pH 7.4), allowing the cells to detach from the brushes. Cell suspensions were centrifuged, resuspended in PBS, and filtered through polyamide gauze (100-μm mesh opening; Stokvis & Smits BV, Ijmuiden, the Netherlands). The filtrates were pelleted by centrifugation, resuspended in PBS, and stored at −70°C until further analysis. Before cytospin preparation, the frozen samples were thawed for 20 min at room temperature. The cell suspensions were cytocentrifuged at 1500 rpm for 5 min (Shandon Cytospin, Cheshire, United Kingdom) onto polysine microslides (E. Merck Nederland B.V., Amsterdam, the Netherlands). The slides were air dried for 1 h and subsequently fixed either in acetone at −20°C for 20 min (prepared for immunohistochemical analysis) or in methanol:acetic acid (3:1) for 10 min (prepared for micronuclei analysis).

Cotinine Immunoassay. Cotinine was determined in plasma and BAL fluid using a standardized RIA according to the published protocols (53, 54).

Urine Mutagenicity Assay. Concentrated urine samples were assayed in a *Salmonella typhimurium* YG1024 test system in the presence of S9 mix (10% liver S12 fractions from Aroclor-pretreated Sprague-Dawley rats) at three doses (25, 50, and 100 μl/plate) in triplicate, with and without β-glucuronidase (1000 units/plate; Ref. 55). For each sample, the average mutagenic response was taken from all experimental settings. The results were expressed as the number of induced revertants (adjusted for the total volume of urine excreted in 24 h).

GC-MS Detection of 4-ABP-Hb Adducts. Erythrocytes were lysed, Hb was isolated, and 4-ABP-Hb adducts were determined by GC-MS according to published methods (51, 56). Briefly, for quantification purposes 4′-fluoro-4-ABP was used as an internal standard. After derivatization of 4-ABP and 4′-fluoro-ABP with pentafluoropropionic anhydride, negative ion chemical ionization mass spectrometry was applied under the selected ion-monitoring conditions at 295 and 313 *m/z*, respectively. The GC-MS setting consisted of a HP 5890 Series II capillary GC (Hewlett Packard, Portland, OR) coupled with a Jeol SX 102A double-focusing MS of reversed geometry (Jeol, Tokyo, Japan). Quantification was done using standard Hb dialysates with known concentrations of 4-ABP (lower detection limit, 1 ng of 4-ABP/g Hb). The intra- and interassay variations were 10.4 ± 5.2% ($n = 4$) and 22.0 ± 3.5% ($n = 5$), respectively.

^{32}P-Postlabeling Assay for Lipophilic-DNA Adducts. DNA was isolated and assayed for lipophilic-DNA adducts by the ^{32}P-postlabeling assay as described earlier (57). TLC was done on polyethyleneimine-cellulose sheets (Macherey Nagel, Düren, Germany) using traditional urea containing solvent systems. For standardization, control samples of [^{3}H]benzo(*a*)pyrene diol epoxide-modified DNA with known modification levels (1 adduct per 10^7 and 10^8 unmodified nucleotides) were run in all experiments. Quantification was done using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) with a lower detection limit of 1 adduct per 10^9 nucleotides.

^{32}P-Postlabeling Assay for 8-OH-dG Adducts. DNA was assayed for 8-OH-dG adducts by a ^{32}P-postlabeling assay as described earlier (58). To avoid artifacts, which may result from radiation-induced oxidation of guanine, a selective hydrolysis of dGp was achieved by treating depolymerized DNA with 90% trifluoroacetic acid before the ^{32}P-postlabeling reaction (59). TLC was carried out on polyethyleneimine-cellulose sheets applying 1.5 formic acid (pH 1.5), D_2, and 0.6 M ammonium formate (pH 6.0). Autoradiography was performed by using a ^{32}P InstantImager Electronic Autoradiographic System equipped with InstantQuant software (model A2024; Packard, Meriden, CT). The results were expressed as 8-OH-dG/10^5 nucleotides.

Immunohistochemical Peroxidase Assay. Immunohistochemistry of PAH-DNA adducts was performed as described earlier (60). Primary antibody 5D11 was applied as first antibody recognizing benzo(*a*)pyrene diol epoxide-DNA adducts and some structurally related PAH diol epoxide-DNA adducts (61, 62). Ultimately, an immunoperoxidase staining was performed with precipitation of 3,3′-diaminobenzidine as end point. Quantification was done using an Image Processing and Analysis System (Quantimet 500, Leica, Cambridge, United Kingdom) through measuring the relative nucleic density of staining (corrected for cytoplasmic and extracellular density of staining as background) in 200 morphologically well-defined cells/slide (range, 0.00–2.40). Detailed information on the quantification procedure and the examination of specificity and sensitivity of immunoperoxidase staining are available from the work of Nia *et al.* (60).

Micronucleus Assay. The micronucleus assay was performed on cytospin slides of MFCs and SPCs stained according to Feulgen reaction. Briefly, the slides were treated to mild acid hydrolysis for 1 h in a 5 N solution of HCl. After several rinses in distilled water, the slides were immersed in filtered Schiff's reagent (Sigma Chemical Co., St. Louis, MO) for 30 min, washed in distilled water, and left in running tap water for 5 min to intensify the staining of the Schiff's reagent. Finally, the slides were blotted dry and mounted in Eukitt (BDH Chemicals Ltd., Poole, United Kingdom). The frequency of micronuclei (‰) was evaluated by scoring 1000 cells/slide at ×1000. The micronuclei were identified according to standardized criteria (63, 64).

TEAC Assay. TEAC assay was performed as described earlier (65). Briefly, after generation of the long-lived radical anion of ABTS in the presence of a thermolabile azo compound, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), plasma was added, and subsequently, absorbance was monitored at 734 nm over a period of 6 min. The decrease in absorbance after addition of the plasma was plotted on a calibration curve established by application of known concentrations of Trolox,

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Table 2 Summary of paired data of the quantified biomarkers in NAC and placebo groups at pre- and postintervention times

	NAC group Mean ± SE			Placebo group Mean ± SE		
	n	T_0	T_1	*n*	T_0	T_1
Cotinine (plasma) (μg/l)	15	445 ± 57	467 ± 56	16	510 ± 47	469 ± 39
Cotinine (BAL fluid; μg/l)	11	1788 ± 315	3678 ± 1793	14	2527 ± 480	1794 ± 291[a]
Urine mutagenicity (×1000 revertants)	6	154 ± 36	133 ± 18	11	153 ± 24	121 ± 19
4-ABP-Hb adducts (ng/g Hb)	16	0.21 ± 0.02	0.20 ± 0.02	16	0.23 ± 0.02	0.26 ± 0.02
Lipophilic-DNA adducts (PBL; /10^8 nucleotides)	15	1.46 ± 0.1	2.50 ± 0.5	16	1.46 ± 0.2	2.52 ± 0.3[b]
Lipophilic-DNA adducts (BAL cells; /10^8 nucleotides)	10	6.01 ± 0.9	4.25 ± 0.8[c]	10	6.03 ± 0.7	5.9 ± 0.7
8-OH-dG adducts (BAL cells; 10^5 nucleotides)	3	4.9 ± 0.7	1.8 ± 0.3[d,e]	3	4.8 ± 0.5	3.2 ± 0.8
PAH-DNA adducts (MFCs; Nucleic stain density)	12	0.04 ± 0.01	0.06 ± 0.01	14	0.05 ± 0.01	0.05 ± 0.01
PAH-DNA adducts (BMCs; Nucleic stain density)	9	0.06 ± 0.01	0.05 ± 0.01	13	0.06 ± 0.01	0.07 ± 0.01
Micronuclei (MFCs; %)	15	1.3 ± 0.3	0.9 ± 0.3[f]	15	1.2 ± 0.3	1.0 ± 0.2
Micronuclei (SPCs; %)	15	1.3 ± 0.2	0.9 ± 0.2	15	1.4 ± 0.2	1.5 ± 0.2
TEAC (plasma; μM/l)	16	393 ± 14	473 ± 19	16	387 ± 11	450 ± 12[g]
TEAC (BAL fluid; μM/l)	11	2043 ± 254	1806 ± 271	14	1703 ± 203	1777 ± 272

[a] Statistically significant as compared with the placebo group at T_0, $P = 0.05$.
[b] Statistically significant as compared with the placebo group at T_0, $P = 0.01$.
[c] Statistically significant as compared with the NAC group at T_0, $P = 0.05$.
[d] Statistically significant as compared with the placebo group at T_1, $P = 0.03$.
[e] Statistically significant as compared with the NAC group at T_0, $P = 0.003$.
[f] Statistically significant as compared with the NAC group at T_0, $P = 0.05$.
[g] Statistically significant as compared with the placebo group at T_0, $P = 0.008$.

an analogue of vitamin E, as standard. Results were expressed as μM/l Trolox.

Statistical Analyses. Results were expressed as mean ± SE throughout the text. Using the Mann-Whitney *U* test, all variables in the NAC group at pre- or postintervention times were compared with their respective controls in the placebo group. Using the Wilcoxon signed rank test, all variables in the NAC group or in the placebo group at preintervention time were compared with their respective controls at the postintervention time. The Spearman rank correlation analysis was done to explore the relationships between different variables. Statistical significance was considered $P < 0.05$.

Results

Although one individual in the NAC group experienced minor side effects, all 41 participants well-tolerated the NAC/placebo treatment throughout and completed the study. Ultimately, the compliance with the study protocol in the NAC-treated group was good and ranged from 75 to 100% with a mean compliance of 90% ($n = 21$). From the beginning, nine individuals refused to undergo bronchoscopic procedures for BAL, mainly because of the invasiveness of this method. Of 32 volunteers who initially underwent the BAL, another 7 persons withdrew from the second sampling. Because of further technical failures in sampling or assaying, some biomarkers could not be quantified at both pre- and postintervention times, T_0 and T_1, respectively, for all individuals. Thus, only paired data are presented and analyzed throughout. Table 2 summarizes the paired measurements of all biomarkers at T_0 and T_1 in this study.

Cotinine and Urine Mutagenicity. Although in the placebo group the smoking indices were nonsignificantly enhanced compared with the NAC group (Table 1), plasma cotinine levels were comparable between the NAC ($n = 21$) and placebo ($n = 20$) groups at T_0 (441 ± 45 *versus* 495 ± 41 μg/l) and at T_1 (467 ± 56 *versus* 469 ± 39 μg/l). That the subjects did not change their smoking habits during the intervention period was confirmed by the paired data, which did not show any significant difference between pre- and postsampling (Table 2). This is also reflected by the observation that in both the NAC and placebo groups, cotinine levels at pre- and postsampling were significantly correlated (Fig. 2). Cotinine levels in BAL fluid were corrected for volume differences by normalizing values for urea in BAL fluid against urea in plasma. Although in the placebo group a significant correlation was observed between cotinine levels in BAL fluid sampled at T_0 and T_1 ($r = 0.6$, $P = 0.05$), levels were decreased after the intervention. In both the NAC and placebo groups, correlations were found between BAL fluid and plasma cotinine levels at T_0 ($r = 0.5$, $P = 0.08$; and $r = 0.4$, $P = 0.07$, respectively) as well as at T_1 ($r = 0.7$, $P = 0.02$; and $r = 0.5$, $P = 0.08$, respectively).

There were no significant differences in urine mutagenicity levels between the NAC and placebo groups nor between pre- and postsampling (Table 2). Only urine mutagenicity levels in placebo group at postsampling were correlated with the respective plasma cotinine data ($r = 0.6$, $P = 0.05$). Overall, plasma/BAL fluid cotinine data and urine mutagenicity did not correspond with the data derived from the questionnaire on smoking status (number of cigarettes smoked per day, amount of tar/nicotine consumed per day).

4-ABP-Hb Adducts. Overall data showed similar 4-ABP-Hb levels in NAC ($n = 21$) and placebo ($n = 20$) groups at T_0 (0.20 ± 0.02 *versus* 0.23 ± 0.02 ng/g Hb; $P = 0.2$) or at T_1 (0.20 ± 0.02 *versus* 0.26 ± 0.02). Analysis of paired samples could not show any effect of NAC supplementation (Table 2). In both the NAC and placebo groups, 4-ABP-Hb adduct levels at T_1 were significantly correlated with those at T_0 ($r = 0.7$, $P = 0.005$; and $r = 0.5$, $P = 0.05$, respectively). Only in the

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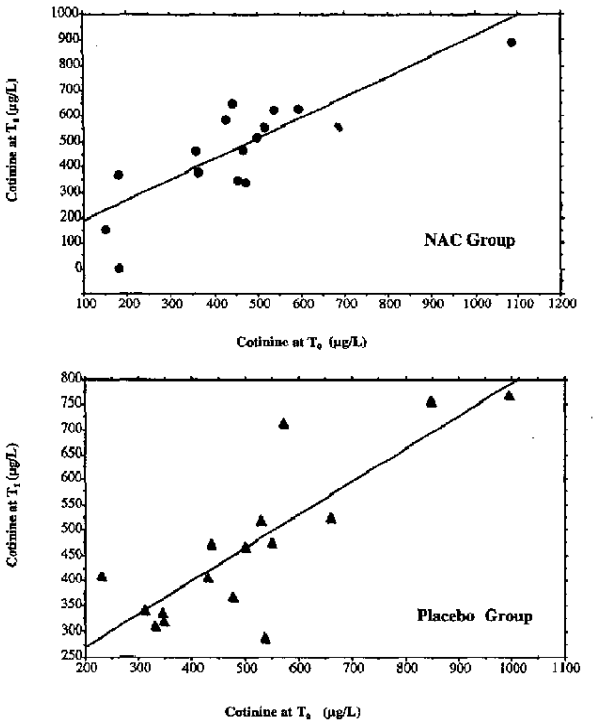

Fig. 2. Correlation between plasma cotinine levels in the NAC ($r = 0.7$, $P = 0.009$) and placebo ($r = 0.7$, $P = 0.005$) groups at time 0 (T_0) and after 6 months (T_1).

placebo group 4-ABP-Hb levels at T_0 correlated with the respective plasma cotinine levels ($r = 0.5$, $P = 0.05$).

DNA Adducts in PBLs and BAL Cells. Briefly, cell yields of the BAL samples at T_0 and T_1 were 10.1 ± 1.2 *versus* 10.9 ± 1.4 × 10^7, $P = 0.9$ in the NAC group, and 9.7 ± 0.9 *versus* 6.8 ± 1.1 × 10^7, $P = 0.02$ in the placebo group. Overall, BAL consisted of pulmonary alveolar macrophages (70–90%) and a minor number of bronchoepithelial cells and lymphocytes.

Qualitatively, there was a typical smoking-associated diagonal radioactive zone in lipophilic-DNA adduct maps of most individuals irrespective of NAC/placebo treatment; no separate adduct spots could be detected (52). Quantitation of the diagonal radioactive zone showed no significant difference in PBL lipophilic-DNA adduct levels between NAC ($n = 21$) and placebo ($n = 20$) groups at T_0 (1.54 ± 1.1 *versus* 1.52 ± 0.14/10^8 nucleotides) or at T_1 (2.50 ± 0.46 *versus* 2.52 ± 0.31). In both the NAC and placebo group, PBL lipophilic-DNA adduct levels were increased at T_1 as compared with T_0 [NAC, 2.50 ± 0.5 *versus* 1.46 ± 0.1, $P = 0.06$ ($n = 15$); placebo, 2.52 ± 0.3 *versus* 1.46 ± 0.2, $P = 0.01$ ($n = 16$)]. No correlation was found between PBL lipophilic-DNA adduct levels at T_1 and the respective levels at T_0, either in the NAC or placebo groups.

There was no significant difference in BAL cell lipophilic-DNA adduct levels between NAC ($n = 12$) and placebo ($n = 16$) groups at T_0 (5.74 ± 0.73 *versus* 6.07 ± 0.60) or at T_1 (4.94 ± 1.03 *versus* 5.61 ± 0.60). Analysis of paired data showed that NAC supplementation significantly decreased lipophilic-DNA adduct levels in BAL cells (6.01 ± 0.9 *versus* 4.25 ± 0.8; $P = 0.05$), whereas in the placebo group the

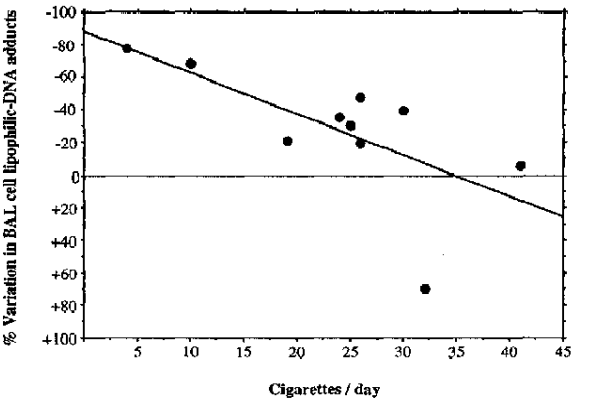

Fig. 3. Dependency of exposure to NAC in modulating BAL cell lipophilic-DNA adducts.

decrease was not significant (6.03 ± 0.7 *versus* 5.90 ± 0.7; $P = 0.7$). Interestingly, the decrease in the NAC group was inversely related to the number of cigarettes smoked/day ($r = 0.7$, $P = 0.04$; Fig. 3). Only in the placebo group was there a significant correlation between BAL cell lipophilic-DNA adduct levels at T_1 and those at T_0 ($r = 0.8$, $P = 0.02$). In the NAC group, BAL cell lipophilic-DNA adduct levels at T_1 were correlated with the respective 4-ABP-Hb levels ($r = 0.8$, $P = 0.01$).

There was no significant difference in BAL cell 8-OH-dG adduct levels between NAC ($n = 5$) and placebo ($n = 4$) groups at T_0 (4.2 ± 0.6 *versus* 4.5 ± 0.5/10^5 nucleotides; $P = 0.6$), however, at T_1 the difference was statistically significant (2.2 ± 0.2 *versus* 3.2 ± 0.4/10^5 nucleotides; $P = 0.03$). Although in both the NAC and placebo groups 8-OH-dG adduct levels were decreased in paired samples after intervention, the decrease was significant only in the NAC group (Table 2). No correlations were observed between 8-OH-dG adduct levels at T_1 and those at T_0. No further interrelations were observed between the respective markers or with self-reported smoking status.

PAH-DNA Adducts in MFCs and BMCs. There was no significant difference in MFC PAH-DNA adduct levels between NAC ($n = 12$) and placebo ($n = 14$) groups at T_0 (0.04 ± 0.01 *versus* 0.05 ± 0.01) or at T_1 (0.06 ± 0.01 *versus* 0.05 ± 0.01). There was also no significant difference in BMC PAH-DNA adduct levels between NAC ($n = 9$) and placebo ($n = 14$) groups at T_0 (0.06 ± 0.01 *versus* 0.06 ± 0.01) or at T_1 (0.08 ± 0.02 *versus* 0.07 ± 0.01). Analysis of paired data of the NAC and placebo groups showed unchanged PAH-DNA adduct levels in MFCs as well as BMCs after intervention (Table 2). No correlations between PAH-DNA adduct levels in MFCs or BMCs at T_1 and those at T_0 was observed. Also, BMC and MFC PAH-DNA adduct levels were not interrelated to each other. Also, there was not any correlation with the respective data on other measurements. Nevertheless, only in the placebo group did PAH-DNA adduct levels correspond with the questionnaire-derived smoking indices for MFCs at T_0 (tar/day, $r = 0.6$, $P = 0.02$; nicotine/day, $r = 0.7$, $P = 0.007$) and for BMCs at T_1 (tar/day, $r = 0.7$, $P = 0.01$; nicotine/day, $r = 0.7$, $P = 0.01$).

Micronuclei Frequency in MFCs and SPCs. There was no significant difference in MFC micronuclei frequency between the NAC and placebo groups, and although in both groups the

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frequency decreased after supplementation, such decrease was significant only in the NAC group (Table 2). There was a significant correlation between MFC micronuclei frequency at T_1 and those at T_0 in both NAC ($r = 0.8$, $P = 0.004$) and placebo groups ($r = 0.6$, $P = 0.02$). Also, no significant difference in SPC micronuclei frequency between NAC and placebo was seen (Table 2). Nor was there any significant difference in SPC micronuclei frequency after supplementation in the NAC group, despite an appreciable decrease (1.3 ± 0.2 *versus* 0.9 ± 0.2, $P = 0.1$; $n = 15$). By combining the data from the two buccal sites, the decrease of micronucleus frequency at T_1 as compared with T_0 was significant in the NAC group (0.9 ± 0.2 *versus* 1.3 ± 0.2; $P = 0.01$). Micronuclei formation data did not correlate with the respective data on other measurements or the smoking status.

Antioxidant Scavenging Capacity of Plasma and BAL Fluid. There was no significant difference in plasma antioxidant scavenging capacity between the NAC ($n = 20$) and placebo ($n = 20$) groups at T_0 (401 ± 14 *versus* 395 ± 9) or at T_1 (451 ± 19 *versus* 450 ± 12). In the NAC and placebo groups, antioxidant scavenging capacity was increased after intervention (Table 2). In both groups, plasma antioxidant scavenging capacity at T_0 and T_1 were correlated (NAC, $r = 0.8$, $P = 0.002$; placebo, $r = 0.7$, $P = 0.01$). There was no significant difference in antioxidant scavenging capacity of BAL fluid between NAC ($n = 14$) and placebo ($n = 17$) groups at T_0 (1856 ± 224 *versus* 1787 ± 176) or at T_1 (1806 ± 271 *versus* 1777 ± 272). Neither in NAC nor in the placebo group was there any significant change in antioxidant scavenging capacity of BAL fluid after intervention (Table 2). Also, there were no correlations between BAL fluid antioxidant scavenging capacity at T_1 and those at T_0. Antioxidant scavenging capacity data did not correlate with the respective data of other measurements or smoking status.

Discussion

A great number of experimental studies have shown the effectiveness of NAC in modulating cancer-associated biomarkers (3, 21, 24–35). However, the chemopreventive effects of NAC in humans have not been verified as yet. Recently, the single large-scale EUROSCAN trial (66) failed to show any efficacy of NAC in preventing or delaying the occurrence of secondary primary tumors in lung or head and neck cancer patients. In the present study, we used a multi-biomarker approach to investigate the chemopreventive effects of NAC in healthy smoking volunteers. The wide array of the investigated markers gave us a unique opportunity to examine the effects of NAC on multiple biological end points as well as to explore the mode of action of NAC because it has been shown that NAC can exert its effects through a variety of coordinated mechanisms (20, 44, 67, 68).

Quantification of exposure dose makers showed no significant change in the level of plasma/BAL fluid cotinine or urine mutagenicity before and after intervention. This was of importance because it confirmed the compliance of the participants with the study protocol and in particular their consistency in smoking behaviors. It should be acknowledged that among the three biomarkers, plasma cotinine was the most relevant exposure index because there was a significant correlation between its analyses at pre- and postintervention times, given that the exposure variable (smoking status) was unchanged throughout.

Assessment of biologically effective dose markers showed that NAC could selectively modify some of these biomarkers in specific cells. In fact, NAC significantly inhibited the formation of both lipophilic-DNA adducts and 8-OH-dG adducts in BAL cells, whereas it had no effect on MFC/BMC PAH-DNA adducts, PBL lipophilic-DNA adducts, and 4-ABP-Hb adducts. Occurrence of these protective effects in humans is in agreement with the findings of animal studies (30–34). Indeed, in rats exposed whole-body to mainstream cigarette smoke, oral NAC attenuated the formation of DNA adducts, as detected by synchronous fluorescence spectrophotometry, in lung, heart, aorta, and kidney (32, 34). In the same animals, NAC modulated the liver metabolism of mutagens (30) and prevented the histopathological and cytogenetic damage produced by cigarette smoke (31). Also, an additional study in rats exposed whole-body to environmental cigarette smoke showed the ability of NAC to inhibit 8-OH-dG induction in lung DNA and formation of DNA adducts detected by ^{32}P-postlabeling in BAL cells, tracheal epithelium, lung, and heart (31). In contrast, in another laboratory oral NAC was found to be ineffective in influencing the levels of DNA adducts in trachea, lung, heart, and bladder (69). Comparative analyses, however, showed that this discrepancy is methodological in nature and, in particular, is attributable to the different chromatographic conditions in D_3.[3] Apparently, the system in which NAC is ineffective grossly underestimates the overall genotoxic potential of cigarette smoke.

Moreover, the efficacy of NAC in an individual cell type is in good agreement with the findings of previous pharmacokinetic studies in animal models reporting a tissue specificity for the uptake and efficacy of NAC (70, 71). Presumably, NAC is deacetylated to L-cysteine in those organs that possess the required metabolic machinery for its deacetylation, thereby boosting the intracellular biosynthesis of GSH or adjusting its turnover rate (3, 20, 72). Although it is still unclear whether deacetylation of NAC within the organ is a prerequisite for its efficacy, it is more likely to observe NAC effects in an organ where it is deacetylated rather than in a nonmetabolizing organ. For example, while being taken up by bladder in a significant amount and only slightly by lymphocytes, NAC exhibited protective effects against the urotoxicity of cyclophosphamide but not against the concomitant leukopenia (73, 74). This might explain our observed inhibitory effects of NAC on the formation of DNA adducts only in BAL cells, provided that this cell type is extensively capable of metabolizing xenobiotics (75–77). The selective inhibition by NAC of DNA adducts in BAL cells and not in PBL or buccal cells could also be ascribed to the higher sensitivity and accessibility of BAL cells to adduct-inducing agents in cigarette smoke. Interestingly, the higher levels of lipophilic-DNA adducts in BAL cells (>4-fold) as compared with those in PBLs, shown in the present study, support this idea.

It is also worth mentioning that the effectiveness of NAC in down-regulating BAL cell lipophilic-DNA adducts was dose dependently related to exposure to cigarette smoke because NAC exhibited the highest efficacy at the lowest exposure level. This is of relevance particularly in the data analysis of chemoprevention trials because influential variables such as exposure, if not taken into account, can easily mislead the investigators when drawing the appropriate conclusions. It is also noteworthy that in the case of the inefficacy of NAC, there was an elevation of most biomarkers over time as adduct levels increased in MFCs, PBLs, and Hb after 6 months of treatment.

[3] A. Izzotti *et al.*, unpublished data.

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We assume that the experimental time course of the study might have caused this phenomenon because it has been shown that all of these markers can vary seasonally because of ambient exposure fluctuations (78–80).

Measurement of biological response markers further reaffirmed the above-mentioned tissue-specific efficacy of NAC as well as provided suggestive evidence on the diversity of the mode of action of NAC. As mentioned earlier, there is a rationale of the efficacy of NAC in the organ where it is metabolized. The uncertainty, however, arises because the metabolized NAC in a given organ, *e.g.*, the liver, may also be taken up by other organs, *e.g.*, by blood circulation (70). In addition, metabolization of NAC or uptake of its metabolites might not necessarily warrant its effectiveness because NAC may exert varying effects through diverse pathways in an individual organ (20, 44, 64, 70). For instance, in rats, when administered p.o., NAC was mainly metabolized in the intestinal mucosa; although its metabolized form could be detected substantially at this site as well as in the bone marrow, there was no modulation of GSH at either site (72, 73). In our study, assessment of the biological response markers showed an inhibitory effect of NAC on the formation of micronuclei in buccal cells, which is in agreement with the anticlastogenic properties of NAC in animal models (64). Moreover, NAC stimulated the antioxidant scavenging capacity in plasma but not in BAL fluid. By comparing this assessment with that of biologically effective dose markers, we found a dual efficacy of NAC within both MFCs and BAL cells; whereas NAC significantly decreased the frequency of micronuclei in MFCs, it did not affect the formation of DNA adducts in the same cell type. Likewise, NAC did not stimulate BAL fluid antioxidant scavenging capacity, whereas it inhibited the formation of DNA adducts in BAL cells. Taken together, along with the findings of previous studies, our observations support the view that NAC may exert varying effects through different pathways of action, thereby exhibiting simultaneous efficacy and inefficacy for various biomarkers in a given organ.

In conclusion, we have demonstrated that NAC can modulate certain smoking-associated biomarkers within specific cells in humans. These results are in agreement with the conclusion of other Phase II chemoprevention trials performed with NAC. Thus, in a follow-up of smokers, the oral administration of NAC (600–800 mg) produced a rapid and significant decrease of urine mutagenicity (81). However, not all of the investigated subjects responded to treatment, which may explain the lack of significant differences between the NAC group and the placebo group observed in the present study. This aspect warrants further studies aimed at understanding the mechanisms that discriminate responders from nonresponders. Individuals respond differently to carcinogenic exposures or drug treatments because genetic polymorphisms in drug- and carcinogen-metabolizing enzymes exist within the population (82). For instance, smokers carrying the *GSTM1* null genotype, and thereof lacking the detoxifying enzyme glutathione-*S*-transferase M1, may have higher DNA adduct levels and are at an enhanced risk for lung cancer (82). It is tempting to speculate that these persons may benefit the most from NAC supplementation. A study in nonsmoking patients suffering from alveolar pulmonary fibrosis showed that the daily administration of three oral doses of 600 mg of NAC resulted in a significant decline of 4-ABP-Hb adducts over time (83). Furthermore, the daily administration of 800 mg of NAC for 12 weeks to patients with previous adenomatous colonic polyps produced a significant decrease of a proliferative index in the colon (84).

It is evident that there is a discrepancy between the protective effects of NAC, as observed in the majority of studies evaluating either preneoplastic conditions and tumors in animal models or biomarkers in both humans and experimental animals, and the lack of effect of NAC in the EUROSCAN Phase III chemoprevention trial (66). It should be noted that these studies have differentiated targets. In fact, Phase II trials are conducted in a primary prevention setting because the recruited subjects are apparently healthy in terms of neoplastic diseases. Similarly, animal studies mimic a primary prevention situation. In contrast, the EUROSCAN study had the main objective of evaluating the occurrence of second primary tumors in patients curatively treated for head and neck and lung cancer. Because NAC has been shown recently, both in *in vitro* and animal models, to possess anti-invasive and antimetastatic properties (85) as well as strong antiangiogenic effects (86), it would be of interest to evaluate possible protective effects of NAC in the context of clinical trials in which the drug should be administered concomitantly with traditional therapy, either surgical or pharmacological or radiological. Studies in mice have in fact shown that NAC and a typical cytostatic drug, doxorubicin, act synergistically in inhibiting the growth of primary tumors and the spread of metastases (87).

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